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van Vugt, B.M.A.

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## **part II**

# **USING SITE-DIRECTED AND RANDOM MUTAGENESIS FOR THE GENERATION OF HIGHLY ACTIVE DRUG METABOLISING MUTANTS OF CYTOCHROME P450 BM3**

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## Evaluation of alkoxyresorufins as fluorescent substrates for Cytochrome P450 BM<sub>3</sub> and site-directed mutants

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BARBARA M. A. VAN VUGT-LUSSENBURG, LLOYD C. BABEL,  
NICO P. E. VERMEULEN AND JAN N. M. COMMANDEUR

adapted from *Analytical Biochemistry* 341 (2005) 148-155

# **ABSTRACT**

In this study, the first fluorescent assay for bacterial cytochrome P450 BM3 (P450 BM3) and mutants is described. P450 BM3 mutants are potentially very versatile biocatalysts for the production of fine chemicals. A fluorescent assay would be very useful for the identification of non-natural ligands in high throughput inhibition assays. Because of the ease and sensitivity of alkoxyresorufin O-dealkylation assays, four different alkoxyresorufines were evaluated as substrates. Wild-type P450 BM3 showed extremely low activity towards all four alkoxyresorufins tested. Five different P450 BM3 mutants were constructed carrying different combinations of mutations R47L, F87V and L188Q, which were previously shown to increase activity towards non-natural substrates. For all mutants, a high benzyloxyresorufin O-dealkylation (BROD) activity was found. The triple mutant P450 BM3 R47L/F87V/L188Q showed highest activity, being 900-fold increased compared to wild-type P450 BM3. The BROD assay could also be applied in whole *E. coli* cells; permeabilisation by LPS-deficiency strongly increased the activity. To demonstrate the applicability of the BROD assay to screen for novel ligands of P450 BM3 R47L/F87V/L188Q, a library of 45 drug-like compounds was tested for inhibition. Eight compounds showed strong inhibition of the BROD-activity, demonstrating for the first time that also drug-like molecules can bind with high affinity to P450 BM3 mutants.

## ALKOXYRESORUFINS AS FLUORESCENT SUBSTRATES FOR P450 BM3

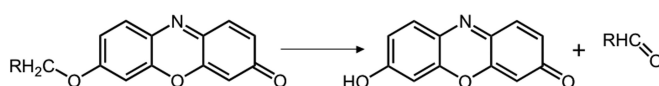
### INTRODUCTION

Cytochromes P450 (P450s) constitute a family of monooxygenases involved in the oxidation and reduction of a large variety of endogenous and exogenous compounds [1-3]. Because of their catalytic diversity and broad substrate range, there is an increasing interest to use P450s as biocatalysts in the production of fine chemicals, including pharmaceuticals [4-6]. Biocatalysis could be used for the discovery of new drugs, or the optimisation of lead compounds and existing drugs. Because of their low stability and catalytic activity, mammalian P450s are not very suitable as biocatalysts [7-9]. Bacterial P450s are often much more stable and exhibit higher catalytic activities, which make them more promising candidates for biocatalysis. The soluble Cytochrome P450 BM3 (P450 BM3) from *Bacillus megaterium* [10] has the highest catalytic activity ever detected for a P450 monooxygenase [7]. This can be explained by the fact that P450 BM3 is a fusion protein, consisting of a catalytic heme-containing domain fused to an FAD and FMN containing reductase domain, which results in very efficient electron transport. Wild-type P450 BM3 catalyses the hydroxylation of long-chain fatty acids at subterminal positions ( $\omega-1$  to  $\omega-3$ ) [10]. However, it has been shown that by mutagenesis of the active site the substrate specificity of P450 BM3 can be engineered to include alkanes [11-13], indole [14], short- and medium chain fatty acids [15, 16] and polycyclic aromatic hydrocarbons (PAHs) [17, 18] as substrates.

For the use of P450 BM3 as a biocatalyst in the pharmaceutical industry, mutants of P450 BM3 are required that are able to bind and metabolise drug-like molecules. At present, such mutants have not been described. The search for such variants is only feasible when fast, sensitive assays are available that allow screening for P450 BM3 mutants that are able to bind drug-like compounds. At present, the only continuous activity assay for P450 BM3 is a colorimetric assay using p-nitrophenoxy-derivatives of long-chain fatty acids as substrates (p-nitrophenoxy-carboxylic acids, pNCAs), which are converted by P450 BM3 into the yellow p-nitrophenolate [19]. A disadvantage of colorimetric assays, however, is that they are generally much less sensitive than fluorescent assays, and more prone to interference by assay components. Therefore, the aim of this study is to develop a fluorescent high-throughput assay for P450 BM3.

As potential substrates, a series of alkoxyresorufins was chosen. Several 7-alkoxyresorufins are known to be fluorescent substrates for mammalian drug metabolising P450s [20-22]. They can be O-dealkylated to yield resorufin, which has an extremely strong fluorescence at very high wavelengths [23] (FIGURE 1). Because of the high sensitivity and the ease of the alkoxyresorufin O-dealkylation assay, these substrates are often used as marker substrates to determine the P450 activity in tissues [20, 24, 25]. Recently, reports have been published on alkoxyresorufins

that are used for high-throughput P450 screening purposes [26, 27]. In addition to their favourable fluorescence properties, another advantage is that alkoxyresorufins can be considered drug-like molecules. pNCAs, on the other hand, bear more structural resemblance to the natural substrates of P450 BM3, i.e. fatty acids. This makes alkoxyresorufins more suitable to screen for P450 BM3 mutants that are able to bind drug-like molecules.



**FIGURE 1:** Alkoxyresorufin O-dealkylation reaction scheme. Methoxyresorufin:  $R = H$ ; ethoxyresorufin:  $R = CH_3$ ; pentoxyresorufin:  $R = C_4H_9$ ; benzyloxyresorufin:  $R = C_6H_5$ .

In this study, we tested four different alkoxyresorufins for metabolism by P450 BM3 and several site-directed mutants. Based on literature data, three amino acid residues were chosen for mutagenesis in order to construct mutants with increased activity towards non-natural substrates. The selected mutations have been shown to enable P450 BM3 to convert PAHs and indole [14, 17, 18], compounds that share some structural characteristics with alkoxyresorufins. The bulky aromatic residue F87, which is positioned above the porphyrin plane [28], prevents the binding of large substrates near the catalytic centre. This residue was therefore replaced by valine. Residue R47 that is located in the entrance channel of P450 BM3 and is known to be involved in substrate recognition was converted to a hydrophobic leucine, to improve the recognition of hydrophobic substrates and to avoid repulsion of basic compounds. Furthermore, the hydrophobic L188, also located in the entrance channel, was replaced by a glutamine. Five different P450 BM3 mutants were constructed containing one, two or all of these mutations. The alkoxyresorufin showing highest activity in cell lysates was also tested in whole *E. coli* cells expressing the triple mutant of P450 BM3, R47L/F87V/L188Q.

## MATERIALS AND METHODS

### MATERIALS

The alkoxyresorufins were purchased from Sigma-Aldrich. The plasmid pT1-P450BM3 containing the P450 BM3 cDNA under control of the temperature-inducible  $P_{R^*P_L}$  promoter was a kind gift from R.D. Schmid (Institut für Technische Biochemie, Universität Stuttgart). The bacteriophage U3 was a kind gift from M. Kranendonk (Department of Genetics, Faculty of Medical Sciences, Universidade Nova de Lisboa). All other chemicals were of analytical grade and obtained from standard suppliers.

### SITE-DIRECTED MUTAGENESIS

Five different site-directed mutants of P450 BM3 were constructed: R47L, R47L/F87V, R47L/L188Q, F87V/L188Q and R47L/F87V/L188Q. The mutations were introduced into pT1-P450BM3

using the QuikChange XL Site-Directed mutagenesis kit (Stratagene). The sequences of the forward primers, with the altered residue in ***bold italic***, were as follows:

R47L 5'-C GAG GCG CCT GGT ***TTA*** GTA ACG CGC TAC TTA TC-3'

F87V 5'-GCA GGA GAC GGG TTA ***GTA*** ACA AGC TGG ACG-3'

L188Q 5'-GAA GCA ATG AAC AAG ***CAG*** CAG CGA GCA AAT CCA G-3'

After mutagenesis, the presence of the desired mutations was confirmed by DNA sequencing (Baseclear, Leiden, The Netherlands).

### EXPRESSION AND ISOLATION OF P450 BM3

The plasmids of the wild-type (pT1-P450BM3) and mutants were transformed into *Escherichia coli* strain DH5 $\alpha$  using standard procedures. Expression was carried out in 3 L flasks containing 300 ml Terrific Broth (TB) medium (24 g/L Yeast Extract, 12 g/L Tryptone, 4 ml/L Glycerol) with additives (1 mM  $\delta$ -aminolevulinic acid, 400  $\mu$ l/L trace elements [29], 0.5 mM thiamine, 100  $\mu$ g/ml ampicillin). Cultures were inoculated with 1/50 volume of an overnight culture in LB medium (5 g/L Yeast Extract, 10 g/L Tryptone, 5 g/L NaCl). Cultures were grown for approximately 3 h at 37 °C and 175 rpm. When the OD<sub>600</sub> had reached 0.8, expression was induced by increasing the temperature to 42 °C. Expression was allowed to continue for 5 h before the cells were harvested. P450 contents of the cells were determined with a CO difference spectrum to determine the expression level [30]. Cells were pelleted by centrifugation (15 minutes, 4000 g, 4 °C), and the pellet was resuspended in 15 ml of KPi-Glycerol buffer (100 mM potassium phosphate (KPi) buffer pH 7.4, 10% glycerol, 0.25 mM EDTA, 0.1 mM DTT). Cells were disrupted using a French Press (1000 psi, 3 repeats). The cytosolic fraction was separated from the membrane fraction by ultracentrifugation in a Beckmann 50.2Ti rotor (45 minutes, 120,000 g, 4 °C). The P450 BM3 containing supernatant was stored at -20 °C, after the P450 contents were determined with a CO difference spectrum.

### ISOLATION OF A LIPOPOLYSACCHARIDE DEFECTIVE (LPS<sup>d</sup>) MUTANT OF *E. COLI* DH5 $\alpha$

To determine the influence of cell wall permeability on the applicability of the BROD assay in whole bacterial cells, permeable mutants of *E. coli* DH5 $\alpha$  were isolated. An LPS<sup>d</sup> mutant of *E. coli* DH5 $\alpha$  was isolated by the bacteriophage U3<sup>r</sup> selection method as described previously [31]. 3 ml of LB medium was inoculated with 30  $\mu$ l of an overnight culture of DH5 $\alpha$  cells. Cells were placed at 37 °C and 200 rpm until the OD<sub>600</sub> reached 0.1. At that point, 100  $\mu$ l of cells were mixed with 100  $\mu$ l bacteriophage U3 phagic suspension. The cells were incubated for 20 minutes at 37 °C with mild agitation. Subsequently, the infected cells were mixed with 2.5 ml of LB top-agar and spread on an LB agar plate. Several colonies were dissolved in a sterile solution 0.85% NaCl and a droplet was plated on a MacConkey agar plate. Colonies that repeatedly (three times) did not grow on MacConkey agar were identified as stable LPS<sup>d</sup> mutants. The plasmid carrying the P450 BM3 R47L/F87V/L188Q gene was transformed into an LPS<sup>d</sup> mutant of *E. coli* DH5 $\alpha$  using standard procedures but leaving out the heat-shock step in the transformation protocol. Expression was performed as described above, with exception of the temperature of induction, which was lowered from 42 °C to 40 °C.



#### DETERMINATION OF ALKOXYRESORUFIN O-DEALKYLATION

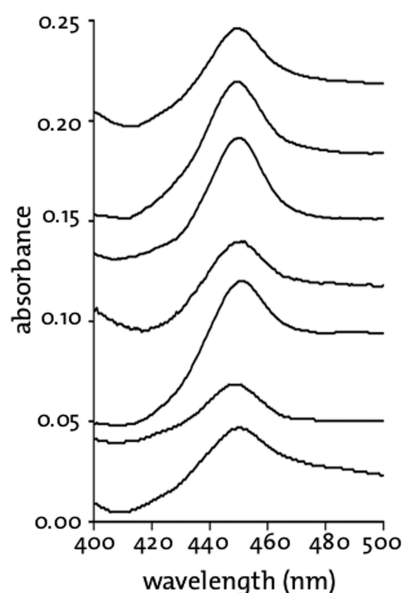
The enzyme activity of P450 BM3 wild-type and the mutants toward a series of alkoxyresorufins was determined in a microtiter plate assay in which formation of resorufin is followed continuously. Reactions were carried out in black Costar 96-well plates in a final volume of 200  $\mu$ l. The assay buffer consisted of 100 mM potassium phosphate (KPi), pH 7.6, supplemented with 5 mM  $MgCl_2$ . Various amounts of cytosolic fraction resulting in enzyme concentrations of 100, 50 and 10 nM were incubated with 10  $\mu$ M of methoxy-, ethoxy-, pentoxy- or benzyloxyresorufin for wild-type P450 BM3 and five site-directed mutants. Reactions were initiated by the addition of an NADPH regenerating system resulting in final concentrations of 0.1 mM NADPH, 0.3 mM glucose-6-phosphate and 0.4 units/ml glucose-6-phosphate dehydrogenase. The reaction was monitored for 15 minutes at 24 °C on a Victor<sup>2</sup> 1420 multilabel counter (Wallac) ( $\lambda_{ex}$  = 530 nm,  $\lambda_{em}$  = 580 nm). The product of the reaction, resorufin, was quantified using a standard curve of the reference compound (5 nM to 500 nM).

#### CHARACTERISATION OF BROD ACTIVITY

Because screening of the alkoxyresorufins showed that benzyloxyresorufin was a good substrate for the triple mutant R47L/F87V/L188Q, the enzyme characteristics of this reaction were investigated in more detail. The assay was characterised by end point measurements that were performed in Eppendorf tubes at 24 °C. The assay buffer consisted of 100 mM potassium phosphate (KPi), pH 7.6, supplemented with 5 mM  $MgCl_2$ . To determine the linearity of product formation in time and the enzyme concentration dependency, 2  $\mu$ M benzyloxyresorufin was incubated for various amounts of time with varying enzyme concentrations (1.25 – 15 nM) in a final volume of 200  $\mu$ l. For the determination of enzyme kinetics, 2.5 nM of enzyme was incubated with benzyloxyresorufin concentrations ranging from 0 to 6.75  $\mu$ M (11 concentrations). Reactions were always initiated by the addition of an NADPH regenerating system as described above, and were allowed to proceed for 4 minutes. The reaction was stopped by the addition of 16  $\mu$ l of 2.5 M trichloro acetic acid (TCA), and subsequently the samples were neutralised by the addition of 200  $\mu$ l of 2 M Tris. 50  $\mu$ l Aliquots were analysed using flow-injection using a Gilson 234 autoinjector coupled to a fluorescence detector ( $\lambda_{ex}$  = 530 nm,  $\lambda_{em}$  = 586 nm). The mobile phase consisted of 50% methanol and had a flow rate of 0.4 ml/minute.

#### HIGH-THROUGHPUT INHIBITION SCREENING

After optimising the BROD assay conditions, its applicability to identify new ligands was tested with a selection of 45 known drug-like ligands for mammalian P450s. The screening assay was performed in black Costar 96-well plates in a final volume of 200  $\mu$ l. The reaction mixture was composed as described above for the batch assay. 2.5 nM of P450 BM3 R47L/F87V/L188Q and 2  $\mu$ M of benzyloxyresorufin were incubated with 50 and 250  $\mu$ M of each library compound. The reaction was monitored for 15 minutes at 24 °C on a Victor<sup>2</sup> 1420 multilabel counter (Wallac) ( $\lambda_{ex}$  = 530 nm,  $\lambda_{em}$  = 580 nm).



**FIGURE 2:** CO difference spectra of wild-type P450 BM3 and site-directed mutants. Spectra were taken on whole *E. coli* DH5 $\alpha$  cells and *E. coli* DH5 $\alpha$  LPS<sup>d</sup> cells as described previously [30]. Spectra represent from bottom to top: wild-type P450 BM3; R47L; R47L/L188Q; R47L/F87V; F87V/L188Q; R47L/F87V/L188Q; R47L/F87V/L188Q in LPS<sup>d</sup> cells.

### BROD ASSAY IN WHOLE CELLS

To determine if BROD activity could also be measured in whole *E. coli* cells, a test was performed in a microtiter plate in a final volume of 200  $\mu$ l. After culturing as described above, *E. coli* DH5 $\alpha$  cells expressing P450 BM3 R47L/F87V/L188Q were pelleted (15 minutes, 4000 g, 4  $^{\circ}$ C) and resuspended in one volume of assay buffer. 20  $\mu$ l of these cells was added to the sample well. An NADPH regenerating system as described above was added. Reactions were initiated by the addition of benzyloxyresorufin resulting in a final concentration of 5  $\mu$ M. The reaction was monitored for 15 minutes at 24  $^{\circ}$ C on a Victor<sup>2</sup> 1420 multilabel counter (Wallac) ( $\lambda_{\text{ex}}$  = 530 nm,  $\lambda_{\text{em}}$  = 580 nm). In addition, the same experiment was performed with a lipopolysaccharide defective (LPS<sup>d</sup>) mutant of *E. coli* DH5 $\alpha$  expressing P450 BM3 R47L/F87V/L188Q that was isolated and cultured as described above.

## RESULTS

### EXPRESSION OF P450 BM3

All five mutants were successfully expressed in *E. coli* DH5 $\alpha$  cells, and the triple mutant was also expressed in DH5 $\alpha$  LPS<sup>d</sup> cells. All expression levels were comparable to those of wild-type P450 BM3. For all the expressed enzymes a typical culture yielded 300-400 nM of enzyme, and no P420, the inactive form of P450, could be detected (FIGURE 2). This shows that the presence

of the mutations did not influence the enzyme expression levels or the stability. After the isolation procedure, the cytosolic fractions contained approximately 6-8  $\mu\text{M}$  of P450 BM3, which indicates that the yield of the isolation procedure was 75-100%.

#### DETERMINATION OF ALKOXYRESORUFIN O-DEALKYLATION

First, the four selected alkoxyresorufins (methoxy-, ethoxy-, pentoxy- and benzyloxyresorufin) were incubated at a concentration of 10  $\mu\text{M}$  with wild-type P450 BM3 and the five mutants. As can be seen in **TABLE 1**, methoxyresorufin was not metabolised by any of the enzymes studied, while ethoxyresorufin was metabolised only at a very low rate by the triple mutant (R47L/F87V/L188Q). With pentoxyresorufin, very low activity was observed for the triple mutant and for double mutant R47L/F87V. Interestingly, benzyloxyresorufin appeared to be a substrate for all six enzymes tested (**TABLE 1**) although for wild-type P450 BM3 and mutant R47L the rate of metabolism was extremely low (0.005 nmol resorufin/min/nmol enzyme). An extremely high BROD activity of 4.5 nmol resorufin/min/nmol enzyme was found for the triple mutant, corresponding to a 900-fold increase compared to wild-type P450 BM3.

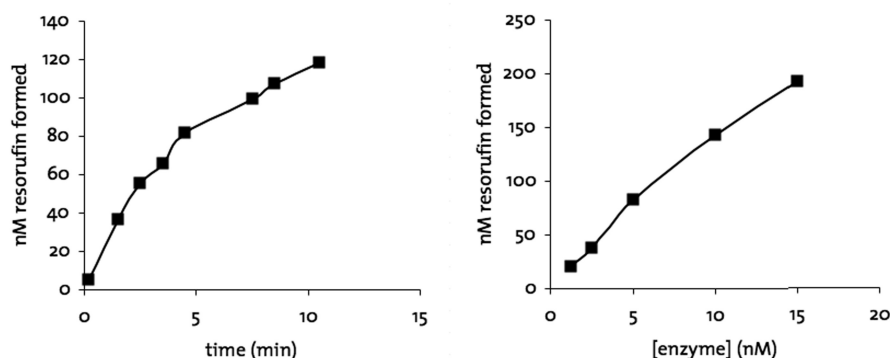
**TABLE 1:** Alkoxyresorufin O-dealkylation activity of P450 BM3 and five mutants.

	V MROD	V EROD	V PROD	V BROD
Wild-type	-	-	-	0.005
R47L	-	-	-	0.005
R47L/L188Q	-	-	-	0.20
R47L/F87V	-	-	0.20	0.71
F87V/L188Q	-	-	-	1.44
R47L/F87V/L188Q	-	0.012	0.22	4.48

*V is expressed in nmol resorufin/min/nmol enzyme. Substrate concentrations were 10  $\mu\text{M}$ . Enzyme concentrations were 100 nM for wild-type P450 BM3 and R47L, and 10 nM for the other mutants. M-, E-, P- and BROD: methoxy, ethoxy, pentoxy and benzyloxyresorufin O-dealkylation.*

#### CHARACTERISATION OF BROD ACTIVITY

The triple mutant R47L/F87V/L188Q was characterised further with respect to BROD activity. Because sample mixing and reaction temperature in the microplate reader cannot be controlled as accurately as in Eppendorf tubes, the BROD assay was characterised using standard batch incubations. First, the ranges were determined where the BROD activity was linear with enzyme concentration and incubation time. The product formation was almost linear with time for up to 4.5 minutes before the activity started to level off (**FIGURE 3A**). Product formation was almost linear with enzyme concentration up to 5 nM (**FIGURE 3B**); at higher enzyme concentrations the product formation leveled off. Based on these data,



**FIGURE 3:** Time- and enzyme concentration dependence of benzyloxyresorufin metabolism. **A:** time dependence of BROD by 2.5 nM P450 BM3 triple mutant. **B:** enzyme concentration dependence of BROD with incubation times of 4 minutes.

**TABLE 2:** Kinetic parameters of the P450 BM3 triple mutant for BROD activity.

	$K_m$ ( $\mu\text{M}$ )	$V_{max}$	$V_{max}/K_m$
R47L/F87V/L188Q	$1.9 \pm 0.1$	$8.6 \pm 1.7$	$4.5 \pm 0.9$
Human P450 3A4 <sup>a</sup>	6.1	0.13	0.021

$V_{max}$  values are expressed in nmol resorufin/min/nmol enzyme. All values are the means  $\pm$  SD of at least three independent measurements. <sup>a</sup>For comparison, literature values of human P450 3A4, for which benzyloxyresorufin is a substrate, are also included [27].

enzyme kinetics analysis of BROD was performed at an enzyme concentration of 2.5 nM of enzyme and with 4 minutes incubation time. Under these conditions  $K_m$  and  $V_{max}$  values of 1.9  $\mu\text{M}$  and 8.6 nmol resorufin/min/nmol enzyme, respectively, were found (TABLE 2).

### HIGH-THROUGHPUT INHIBITION SCREENING

To demonstrate the applicability of the BROD assay to serve as a high-throughput inhibition assay to identify drug-like compounds with affinity for P450 BM3, a library of 45 drug-like compounds (see legend TABLE 3) was tested. Compounds were considered hits when more than 10% inhibition was observed at an inhibitor concentration of 50  $\mu\text{M}$ . As listed in Table 3, eight hits were found. Ketoconazole, tranilcypromine, tamoxifen and hydroxyellipticine were found to be very strong inhibitors. At 50  $\mu\text{M}$ , ketoconazole inhibited BROD activity completely, so for this compound lower concentrations were tested (0.23 nM – 1500 nM). With these data, an  $\text{IC}_{50}$  value of 21.3 nM was found (FIGURE 5). Orphenadrine, proadifen and quinidine inhibited BROD activity for approximately 50% under the conditions used, and carbamazepine displayed a low inhibitory potency of 25%.

**TABLE 3:** Hits found in a library of 45 drug-like compounds.

Compound	% Inhibition at 50 $\mu$ M
Ketoconazole	100
Tranlycypromine	93
Tamoxifen	92
Hydroxyellipticine	91
Orphenadrine	59
Proadifen	57
Quinidine	40
Carbamazepine	25

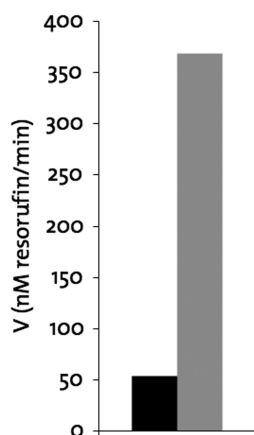
A hit is defined as a compound that inhibits BROD activity of the P450 BM3 triple mutant at an inhibitor concentration of 50  $\mu$ M for >10%. The percentage of inhibition at this concentration is indicated in the table. The compounds in the library that were not picked up as hits were:  $\alpha$ -naphthoflavon, 3-acetamidofenol, adriamycine, aminopyrine, amodiaquine, arterenol, barbital, caffeine, chlorzoxazone, cloxacillin, clozapine, codeine, dacarbazine, debrisoquine, dexamethasone, dextromethorphan, diclofenac, dicoumarol, disulfiram, flucoxacilline, furosemide, indomethacine, ketamine, metyrapone, mianserine, phenobarbital, raloxifen, sparteine, sulfaphenazole, tacrine, theophylline, troleandomycine, xylocaine, nortriptyline, indol.

### BROD ASSAY IN WHOLE CELLS

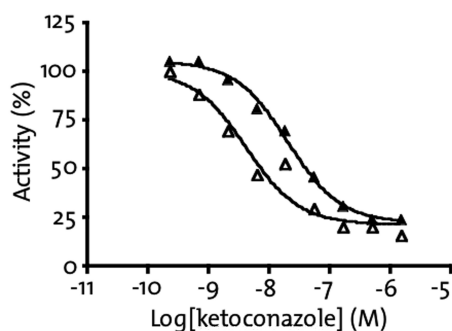
To investigate whether the BROD assay could also be performed in whole cells, *E. coli* DH5 $\alpha$  cells expressing P450 BM3 R47L/F87V/L188Q were incubated with benzyloxyresorufin. The results show that under the conditions used, BROD activity in whole cells is very low (FIGURE 4). Incubation of a lipopolysaccharide defective (LPS<sup>d</sup>) mutant *E. coli* DH5 $\alpha$  cells showed a 7-fold increased BROD activity. This may be explained by the fact that LPS<sup>d</sup> mutants miss a major component of the outer membrane, and thus have an increased permeability for many compounds. The IC<sub>50</sub> value of ketoconazole in LPS<sup>d</sup> cells was determined by adding 0.23-1500 nM of ketoconazole to the assay. This resulted in an IC<sub>50</sub> value of 4.3 nM, which is in the same range as the IC<sub>50</sub> value found using cytosolic fraction (FIGURE 5).

### DISCUSSION

The aim of this study was to develop a fluorescent high-throughput screening method for P450 BM3 that can be used for inhibition screening. Such an assay would be of great value to identify novel ligands that are able to bind P450 BM3 or P450 BM3 mutants. Because of their favourable fluorescence properties and their structural similarity to drug-like molecules, alkoxyresorufins were selected as potential substrates for P450 BM3. Of the four alkoxyresorufins that were tested on wild-type and mutant forms P450 BM3, benzyloxyresorufin appeared to be the best substrate. It was metabolised by all six enzymes to a certain extent. The highest BROD activity



**FIGURE 4:** BROD activity in whole *E. coli* cells. Rates of benzyloxyresorufin metabolism (nM resorufin formed per minute) by the P450 BM<sub>3</sub> triple mutant in normal *E. coli* DH5α cells (black bar) and *E. coli* DH5α LPS<sup>d</sup> cells (grey bar). Enzyme contents of normal cells and LPS<sup>d</sup> cells were similar.



**FIGURE 5:** Dose-response curves of ketoconazole in cytosol and whole cells. Data were obtained by incubation of 0.23–1500 nM ketoconazole and 5 μM benzyloxyresorufin with 2.5 nM P450 BM<sub>3</sub> triple mutant in cytosol (closed triangles) or with 20 μl whole LPS<sup>d</sup> DH5α cells expressing P450 BM<sub>3</sub> triple mutant (open triangles). IC<sub>50</sub> values were 21.3 nM in cytosol and 4.3 nM in whole LPS<sup>d</sup> DH5α cells.

was found for the triple mutant, R47L/F87V/L188Q (TABLE 1). This enzyme displayed a  $V_{max}/K_m$  value over 200-fold higher than that of human P450 3A<sub>4</sub>, for which benzyloxyresorufin is a substrate (TABLE 2). This suggested that benzyloxyresorufin could be a promising diagnostic substrate for P450 BM<sub>3</sub>.

The fluorescent high-throughput BROD assay described in this study has various advantages compared to the assays currently available for P450 BM<sub>3</sub>. One technique frequently used to determine P450 BM<sub>3</sub> activity is the measurement of NADPH consumption [32]. The main disadvantages of this assay are that monitoring of cofactor depletion is far less sensitive than product formation, and that uncoupling of the enzyme can occur which results in false positive signals [1]. Other techniques, based on product identification and quantification with GC-MS, NMR or HPLC, are not suitable for high-throughput purposes because of long analysis

times and/or laborious sample preparations [11, 18]. The only continuous activity assay for P450 BM3, that has already been used to identify P450 BM3 mutants with altered activity, is the p-nitrophenoxy-carboxylic acid (pNCA) assay [13, 15, 33]. The product of the reaction is p-nitrophenolate, which can be detected at 410 nm. Although the  $V_{max}/K_m$  value for 10-pNCA conversion by P450 BM3 F87A is approximately 10-fold higher than that of benzyloxyresorufin metabolism by the triple mutant [15], the detection limit of p-nitrophenolate is approximately 200-fold higher than the detection limit of resorufin used in this study. This makes the spectrophotometric assay much less sensitive than the fluorescent BROD assay. In addition, the high excitation and emission wavelengths of resorufin make the assay less sensitive to interference by other assay components. This is especially important when the assay is used with whole cells instead of crude lysates or purified enzymes.

For applications of the BROD assay for screening of libraries of random P450 BM3 mutants, it is very valuable that the assay can also be performed in whole *E. coli* cells because this makes laborious enzyme isolation procedures obsolete. Recently, it has been shown that methoxyresorufin can be used in whole cell screening assays with *E. coli* cells expressing human P450 1A2 mutants [26]. In the present study, benzyloxyresorufin is efficiently metabolised in whole DH5 $\alpha$  cells. The use of an LPS<sup>d</sup> bacterial host leads to a strong increase in BROD-activity (FIGURE 4), which can be explained by a higher permeability for benzyloxyresorufin. The increased permeability of LPS<sup>d</sup> cells toward chemicals has previously been used to increase the sensitivity of various mutagenicity tester strains [34, 35].

Data in TABLE 1 suggest that for benzyloxyresorufin metabolism the F87V mutation is most important: the double mutant R47L/L188Q that lacks the F87V mutation has a 23-fold lower BROD activity compared to the triple mutant. This confirms the crucial role of F87 in controlling substrate specificity of P450 BM3. The R47L/F87V mutant that lacks the L188Q mutation has a 6-fold lower BROD activity compared to the triple mutant. The effect of the R47L mutation was least pronounced; the double mutant F87V/L188Q lacking this mutation showed only 2-fold decreased BROD activity compared to the triple mutant. Apparently, this residue plays a minor role in binding of benzyloxyresorufin. The F87V/L188Q mutant has already been described in literature; this mutant was shown to metabolise indole [14] and PAHs [17].

Benzyloxyresorufin is a well-established substrate for several human drug metabolising P450s, including P450 3A4. The active sites of P450 BM3 and human P450 3A4 appear to have several features in common. Both human P450 3A4 and P450 BM3 can apparently bind two substrates in the active site simultaneously [36, 37]. Also, both enzymes have a relatively large active site composed of mainly hydrophobic residues [28, 38, 39]. Therefore, it is not surprising that of the four alkoxyresorufins used in this study, the large and hydrophobic benzyloxyresorufin was found to be the best substrate for P450 BM3.

The BROD assay was subsequently used as a high-throughput inhibition screening method. In a library of 45 drug-like compounds, several ligands for P450 BM3 R47L/F87V/L188Q were found (TABLE 3). Three of these compounds can be classified as general P450 inhibitors (ketoconazole, proadifen and tranilcypromine), and four of the eight hits are known substrates of human P450 3A4 (carbamazepine, hydroxyellipticine, quinidine and tamoxifen).

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This is the first report where drug-like molecules have been identified as ligands for P450 BM<sub>3</sub>. Together with the other favourable characteristics of P450 BM<sub>3</sub> with respect to stability and catalytic activity, this makes P450 BM<sub>3</sub> a very good candidate to be used as a biocatalyst for the discovery and synthesis of drugs [4]. It still needs to be investigated, however, whether the drug-like compounds identified as hits in this study are also metabolised by P450 BM<sub>3</sub>. In addition, alternative P450 BM<sub>3</sub> mutants could be constructed that still possess the ability to perform BROD, but have altered activities toward drugs. This could lead to a series of P450 BM<sub>3</sub> mutants that can convert different classes of drug-like compounds into novel metabolites, resulting in libraries of structurally related molecules with potentially therapeutically interesting properties.





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